Histopathological features of the glandular mucosa of the normal equine stomach and comparison with lesions in a horse with equine glandular gastritis-erosion syndrome (EGGES)

Finnhorse during a harness race

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Tiivistelmä - Referat – Abstract

Research on lesions in the equine stomach has concentrated mainly on equine gastric ulcer syndrome (EGUS). Lesions in the glandular area of the antrum and the pylorus, however, have so far received much less attention. The aim of this study was to contribute to the understanding of the histopathology of glandular lesions. The first part of the thesis consists of a thorough review of the literature on this subject. The literature review discusses the relevant literature on aetiology, pathophysiology, diagnosis, treatment and prevention of equine glandular gastritis-erosion syndrome (EGGES). The second part is an empirical study in which the normal histopathology of the glandular mucosa of the equine stomach is examined and described, and then comparisons are made with a horse that has pathological evidence of EGGES. In addition, the gross lesions and the histopathological findings are presented as photographs.

The horses for this study were selected from horses euthanased at the Equine Hospital of the Helsinki University Veterinary Teaching Hospital and sent to the Pathology and Parasitology unit of the Faculty of Veterinary Medicine, University of Helsinki for necropsy. The material was collected from August 2013 – February 2014. Only adult horses were included in this study. Altogether five stomachs were sampled. Two stomachs showed gross lesions and three appeared normal. However, only one normal-looking and one with lesions were suitable to be analysed for study purposes.

The glandular lesions in the stomach with EGGES were shown to be more severe than any described in the literature before. However, the influence of the original illness and treatment of the horse could not be assessed. The study showed that in addition to erosions, hyperaemia, glandular atrophy, dysplasia and hyperplasia described in the literature before, severe ulcers and vasculitis can also be seen in the glandular stomach of horses.

Avainsanat - Nyckelord - Keywords

equine, gastric ulcer, histopathology, hevonen, mahahaava, histologia

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Tiivistelmä - Referat – Abstract

Hevosen mahahaavaa käsittelevä tutkimus on keskittynyt lähes ainoastaan mahan rauhasettoman osan mahahaavaoireyhtymään (equine gastric ulcer syndrome, EGUS). Rauhasalueen muutoksia antrumin ja pyloruksen alueella on tutkittu vain vähän.

Tämän tutkielman tavoitteena oli lisätä tietämystä rauhasalueen muutosten histopatologiasta. Tutkielman ensimmäinen osa on perusteellinen kirjallisuuskatsaus alan tärkeimmästä kirjallisuudesta. Katsauksessa käydään läpi rauhasalueen gastriitti-eroosio-oireyhtymän (equine glandular gastritis-erosion syndrome, EGGES) etiologian, patofysiologian, diagnosoinnin, hoidon ja ehkäisyn kannalta olennainen kirjallisuus. Tutkielman toinen osa on empiirinen tutkimus, jossa kuvaillaan terveen hevosen mahan rauhasalueen histologiaa ja verrataan sitä sellaisen hevosen mahaan, jolla on EGGES. Lisäksi tutkielmassa on valokuvia tärkeistä makroskooppisista ja mikroskooppisista löydöksistä.

Tutkimuksen hevoset valittiin hevosista, jotka oli lopetettu Yliopistollisn eläinsairaalan hevossairaalassa ja toimitettu eläinlääketieteellisen tiedekunnan patologian ja parasitologian osastolle raadonavaukseen. Aineisto kerättiin elokuun 2013 ja helmikuun 2014 välisenä aikana. Vain aikuiset hevoset hyväksyttiin tutkimukseen. Näytteitä otettiin viidestä mahasta, joista kahdessa oli makroskooppisia muutoksia ja kolme näytti terveeltä. Vain kahta näistä mahoista pystyttiin hyödyntämään tutkimuksessa, yhtä tervettä ja yhtä, jossa oli muutoksia.

Tutkitussa mahassa rauhasalueen muutokset olivat vakavampia kuin mitkään aiemmin kirjallisuudessa kuvatut. Hevosen perussairauden ja hoidon vaikutusta muutosten kehitykseen ei pystytty arviomaan. Tutkimus kuitenkin osoitti, että eroosioiden, hyperemian, rauhasten atrofian, dysplasian ja hyperplasian lisäksi myös vakavia haavaumia ja vaskuliittia voi esiintyä hevosen mahan rauhasalueella.

Avainsanat - Nyckelord - Keywords

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Työn johtaja Riitta-Mari Tulamo
Ohjaaja Michael Hewetson

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1 INTRODUCTION

Since the mid-1980's equine gastric ulcers have been a growing area of research. As the awareness of the clinical and economical significance of gastric ulcers, especially in the racing world, has increased during the 1990's and the 21st century, an increasing number of studies have been conducted in this field. Research on lesions in the equine stomach has concentrated mainly on gastric ulcer disease, which due to its multifactorial aetiology is often described as equine gastric ulcer syndrome (EGUS) (Merritt 2009). The focus has been on the squamous part of the stomach as many studies have stated that squamous ulcers are far more common than glandular ulcers (Murray et al. 2001a, Andrews et al. 2002). Even though there are many unknown features in EGUS, it seems clear that the erosions and ulceration of the mucosa are caused by prolonged exposure to gastric acid (Murray 1999). Multiple risk factors have been identified, such as intensive training, fasting and a diet consisting of high grain and low roughage ratios (Nadeau & Andrews, 2009).

Lesions in the glandular area of the antrum and the pylorus, however, have so far received much less attention. Murray et al. (2001b) reported endoscopic findings in the antrum and pylorus of 162 horses between 1996-2000. They used a 3m equine gastroscope to achieve a better view of the antrum and pylorus. Martineau et al. (2009 a, b) conducted a study on the pathology of gastritis and gastric ulceration of the horse at *post mortem*. They made a comprehensive histopathological assessment of both squamous and glandular lesions and attempted to create a scoring system for such lesions. Husted et al. (2010) examined glandular lesions for bacteria including *Helicobacter pylori*. Habershon-Butcher et al. (2012) studied the prevalence of glandular ulcers in Australia and the UK and made an attempt to identify risk factors for glandular ulcers. Most researchers agree that acid exposure does not play a significant role in the formation of glandular lesions, and that further studies are needed to identify the underlying cause/s (Murray et al. 2001b, Husted et al. 2010).

For this study, the term equine glandular gastritis-erosion syndrome (EGGES) was adopted to try and differentiate glandular lesions from EGUS. The term EGGES was

first suggested by Richard Hepburn (2012) to describe the unique endoscopic appearance and pathology of these gastric lesions, and is used to describe a variety of lesions in the glandular mucosa. The aim of this study is to contribute to the understanding of the histopathology of glandular lesions. The first part of the thesis consists of a thorough review of the literature on this subject. The second part is an empirical study in which the normal histopathology of the glandular mucosa of the equine stomach is examined and described, and then comparisons are made with a horse that has pathological evidence of EGGES.

2 LITERATURE REVIEW

2.1 Anatomy of the equine stomach

The horse has a surprisingly small stomach with a capacity of 5-15 litres (Dyce et al. 2010). It has a unique anatomical shape and is positioned in such a way that the cardia and the pylorus lie very close to each other at the minor curvature. At the oesophageal inlet, the stomach has an exceptionally strong cardiac sphincter. The unusually large fundus is called the *saccus caecus*. It is the most dorsal and caudal part of the stomach (Nickel et al. 1995). The outlet at the pylorus is relatively small, consisting of two muscular thickenings: the antral pyloric sphincter at the transition from the pyloric antrum to the pyloric canal and the pyloric sphincter at the pylorus (Nickel et al. 1995, Dyce et al. 2010).

2.2 Histology of the equine stomach

The stomach wall consists of four layers: *tunica mucosa*, *tunica submucosa, tunica muscularis* (or *muscularis externa*) and *tunica serosa*. The *tunica mucosa* can further be divided into *lamina epithelialis mucosae, lamina propria mucosae* and *lamina muscularis mucosae* (Banks 1993, Ross & Pawlina 2011). In the nonglandular part of the stomach, which in the horse comprises the entire *saccus caecus* and part of the body, the mucosa is lined by stratified squamous epithelium. The transition from the nonglandular to the glandular part is clearly visible: at the *margo plicatus* the squamous epithelium turns into simple columnar epithelium (Dyce et al.2010). These columnar cells secrete a thin layer of mucus onto the mucosal surface. This mucus is an important part of the protective barrier of the glandular mucosa. The glandular mucosa is folded. The volume of the stomach content determines the extent of these folds. The mucosa is composed of longitudinal elevations, the gastric areas, and grooves between these elevations, gastric pits. The gastric glands are embedded in the *lamina propria* (Banks 1993, Nickel et al. 1995).

The glandular part can be divided into three regions: the cardiac region, the proper gastric or fundic region and the pyloric region (Dyce et al. 2010). This distinction is based not only on the anatomical relations of these areas but also on the type of cells histologically present in each region. In the horse, the cardiac gland region is a narrow

area close to the *margo plicatus*. Various tubular glands empty into the gastric pits. Mucous glands are the principle type found in the cardiac gland region.

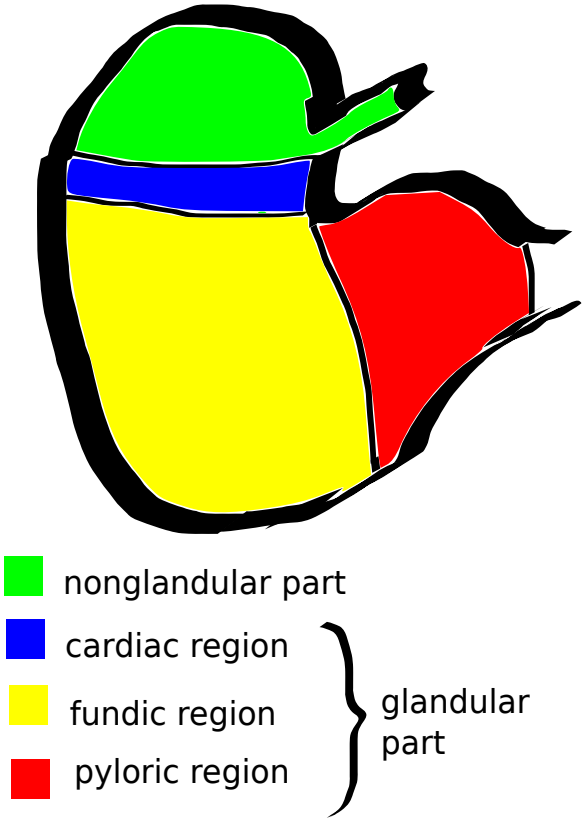

Fig. 1 Histologic division of the equine stomach

The fundic gland region is reddish-brown with histologically clear gastric areas and pits (Nickel et al. 1995). In this area the glands are less frequently branched but longer than in the cardiac region. This makes the lamina propria thicker in this part. In the fundic gland region, glands are constructed mostly of parietal and chief cells, which secrete hydrochloric acid and pepsinogen, respectively. Other cells found in this area are endocrine cells (enterochromaffin-like cells) secreting various hormones such as serotonin, gastrin and histamine (Banks 1993). The pyloric gland region is yellowish to pale red and comprises the pyloric antrum and canal. In this part the gastric pits are deeper than in the cardiac region but in other aspects the pyloric glands resemble the cardiac glands (Nickel et al.1995). The endocrine glands in the pyloric region are the G-cells, the D-cells and the ECL cells. These cells secrete gastrin and somatostatin respectively (Merritt 1999).

2.3 Physiology of the equine stomach

The stomach serves as a temporary depot for food on its way from the oesophagus to the duodenum. In the stomach, the food is processed chemically by gastric juice, which is composed of the products secreted by the stomach glands: hydrochloric acid and pepsinogen (Nickel et al. 1995) and, as more recent research implies, the products of the salivary glands, pancreatic glands, biliary glands and duodenal glands. It is most likely due to the duodenal reflux that gastric pH in horses varies between 1.5 and 7.0 when the stomach is empty. During feeding pH rises by 1 or 2 points depending on the feeding regime (Merritt 1999).

The physiology of the equine glandular stomach is not thoroughly investigated. Most research has been done on dogs and rodents (Merritt 1999) and many concepts adopted from the results of studies in humans. The function of the cardiac region is poorly understood (Merritt 1999). In swine, its glands primarily secrete bicarbonate but whether this applies for the horse is not known. It has been shown that the horse's cardiac region has numerous somatostatin immunoreactive cells, which control the gastric acid secretion endogenously by modulating the gastrin release of G-cells (Merritt 1999).

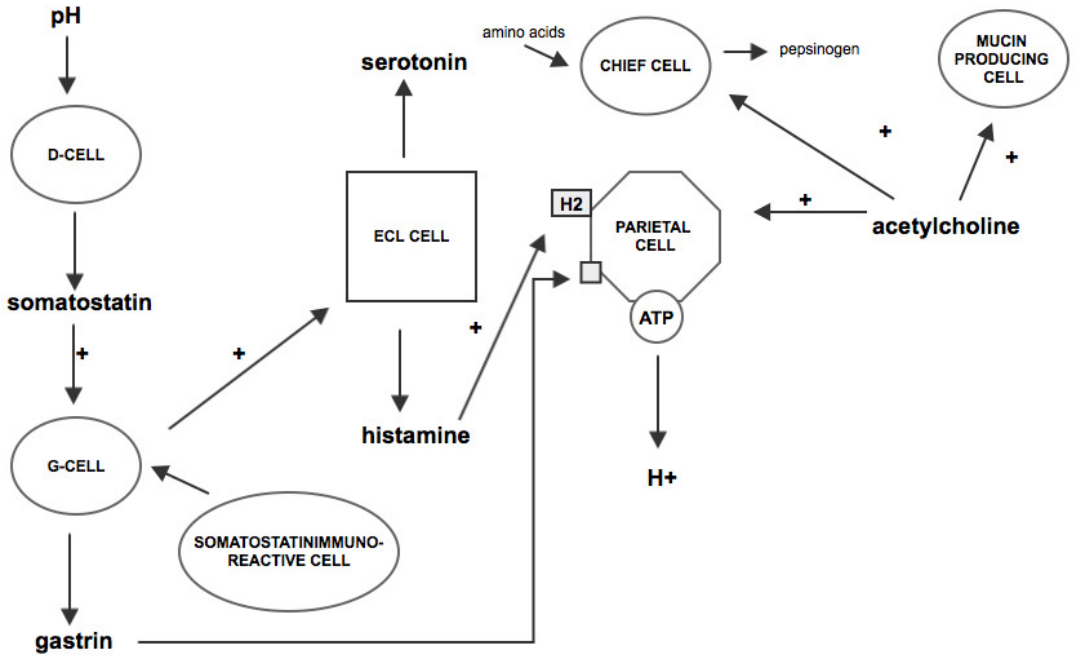

Fig. 2 Simplified diagram showing interaction of cells in glandular mucosa.

In the fundic region are the traditional gastric cells: parietal cells producing hydrochloric acid and chief cells producing pepsinogen. The ECL cells secrete histamine, which stimulates the H_2-receptors on the parietal cells to secrete more HCl. In addition, they secrete serotonin, which controls gastric blood flow and secretion. The G-cells in the pyloric region are the most important source of gastrin in the equine stomach. There are less somatostatin-secreting D-cells in the pyloric than in the fundic region but more serotonin-secreting ECL-cells. The antral mucosal glands are mostly mucus-producing (Merritt 1999).

In humans, the gastric mucosa is protected from gastric acid, pepsin and bile acids by an extracellular mucus barrier consisting of mucus gel, HCO_3^-, and surfactant phospholipids. This barrier keeps the gastric epithelium at a neutral pH, prevents pepsin infiltration and repels water-soluble agents, which might cause damage to the mucosa (Allen & Flemström 2005, Laine et al. 2008).

Should harmful agents such as acids penetrate this barrier they would be faced by another line of defence mechanisms: the epithelial barrier. The surface epithelial cells produce mucus, bicarbonate, prostaglandins and, in case of stress such as acid exposure, heat shock proteins which prevent protein denaturation. They are connected with tight junctions preventing acid and pepsin to diffuse back and have similar hydrophobic properties as the mucus barrier. Continuous cell renewal keeps the mucosa intact and makes sure that minor injuries to the mucosa are repaired within seconds. In humans, the entire gastric mucosa is replaced in 3-7 days but the renewal of the glandular cells takes months (Laine et al. 2008).

Another important protective mechanism is mucosal blood flow. Mucosal capillaries ending in venules provide the epithelial cells with oxygen and nutrients and clear it of toxic substances. The endothelial cells of the microvasculature produce nitric oxide (NO) and prostacyclin (PGI_2). These substances contribute to the viability of the endothelial cells and the continuity of mucosal blood flow as they prevent platelets and leukocytes from adhering to the endothelium. In addition, NO acts as a mediator when sensory nerves stimulated by the entry of luminal content or acid into the mucosa leads to an increase in mucosal blood flow (Laine et al. 2008).

The mucosa generates prostaglandin E_2 (PGE_2) and prostacyclin which stimulate or facilitate almost all the defence mechanisms discussed above. In addition, they inhibit mast cell activation (Laine et al 2008). It has been shown *in vitro,* that PGE_2 is produced by the equine stomach mucosa (Morrissey et al. 2008) but as to the other features of the protective mechanisms, research in equines is scarce. However, there is no reason to believe that the protective mechanisms of the glandular mucosa should differ significantly from those described in humans.

2.4 Equine glandular gastritis-erosion syndrome (EGGES)

For a long time glandular lesions in the equine stomach were considered to be less frequent than squamous ulcers and therefore of less importance. One of the main reasons for this conclusion seems to be the fact that researchers had used a 2-m-long endoscope in the past, which did not allow a complete view of the antrum and pylorus (Murray et al. 2001b), and according to some studies, glandular lesions seem to be located more often in the antrum and the pylorus than in the glandular fundus (Murray et al. 2001b, Husted et al. 2010). Other reasons for missing glandular lesions are an insufficient fasting period which leads to food residue in the stomach (Vatistas et al. 1999), fluid in the stomach, inadequate insufflation of the glandular region and inexperience of the endoscopist (Andrews et al. 2002).

Equine glandular lesions show a wide variety of forms. The literature describes erosions and hyperaemia as well as glandular atrophy, dysplasia or hyperplasia (Martineau 2009a). This variety makes it difficult to compare the numbers for prevalence of glandular lesions found in the studies. Some of the studies have only included glandular erosions and ulcers whereas others have counted all kinds of lesions. In addition, in several studies the pylorus could not be viewed in all horses included in these studies. The prevalence of glandular erosions and ulcers varies between 22-58% (Murray et al. 2001b, Bezdekova & Futas 2009, de Bruijn et al. 2009, Luthersson et al. 2009a, Husted et al. 2010, Tamzali et al. 2011) with several studies reporting a prevalence of 50-60% (Murray et al. 2001b, Begg & O'Sullivan 2003, Luthersson 2009a). Habershon-Butcher et al. (2012) reported an overall prevalence of 50.4% with a higher prevalence in Australia than in the UK.

2.4.1 Aetiology

As there is much less research on glandular lesions than on squamous lesions there is also less literature on the possible aetiology of EGGES. In this chapter, I will try to cover briefly all the different possibilities that have been discussed in connection with both EGUS and EGGES.

Many aspects of EGUS are still unknown but there is an understanding between researchers that increased exposure to acids is the main reason for ulcer formation in the squamous epithelial mucosa of the equine stomach (Lorenzo-Figueras & Merritt 2002, Nadeau et al. 2003). It seems that in addition to hydrochloric acid, the volatile fatty acids produced by bacteria during fermentation may play an important role in ulcer formation at least if gastric pH is low (Nadeau et al. 2000). Another component of the equine gastric juice that may be potentially harmful to the gastric mucosa is bile, as it has been shown that duodenal reflux takes place between feeding times and that the concentrations of bile and acid reach potentially harmful levels after 14 hours of fasting (Berschneider et al. 1999).

It seems clear however, that acid exposure may not be the only reason for EGGES. Firstly, the glandular mucosa is supposed to be in contact with the acidic gastric juice and thus has much better defence mechanisms against acids. Secondly, research has not shown any correlation between the presence of ulcers in the squamous vs. glandular regions (Murray et al. 2001b, Begg and O'Sullivan 2003), which should be the case if the underlying factor was the same. Thirdly, protocols used to provoke squamous ulcers such as feed deprivation have not induced glandular lesions (Murray et al. 2001a). Duodenal reflux, on the other hand, has been discussed as a probable cause for glandular lesions (Murray et al. 2001b). In humans, bile acids have been shown to increase the risk of intestinal metaplasia (Matsuhisa et al. 2013). It would be plausible that this effect would be seen in the pyloric region in particular as it is the region closest to the duodenum where the amount of bile can be expected to be higher than in the rest of the stomach.

The role of bacteria in the aetiology and pathogenesis of glandular lesions has been speculated on (Murray et al. 2001b.). As *Helicobacter pylori* is known to cause

ulceration in man, this and other *Helicobacter* subspecies have been the subject of most research in horses. Moyaert et al. (2007b) found a new *Helicobacter* subspecies in horse faeces which they named *Helicobacter equorum.* However, according to a later study, *H. equorum* neither causes clinical signs nor any macroscopical or microscopical post mortem changes in adult horses (Moyaert et al. 2007a).

Another study was conducted by Contreras et al. (2007) in Venezuela on 20 racehorses euthanased due to open leg fractures. The horses had no history of gastro-intestinal symptoms. Samples were taken from both sides of the *margo plicatus* and after fixation in 100% ethanol DNA was extracted for PCR assays to detect *Helicobacter* DNA. Histopathology showed that 90% of the horses had gastric lesions: 35% had ulcerations and 25% gastritis. *Helicobacter* genus-specific PCR amplicons were found in 55% of the horses studied. Only one of these 11 horses had a healthy stomach mucosa, the others showed either ulcers, gastritis or both in the squamous and glandular mucosae. However, in addition to these 10 horses, lesions were also seen in horses that were negative on PCR for helicobacter. The DNA was more closely related to *H. pylori* than *H. equorum,* but tested negative in *H. pylori*-specific PCR assays. The researchers were not able to isolate the bacteria and stated that further studies on this subject were necessary to determine the pathogenic potential of *Helicobacter spp.* in horses.

The relationship between *Helicobacter* species and gastric ulcers in the glandular mucosa was further investigated by Bezdekova and Futas (2009). Biopsies from the pyloric region of 22 horses with gastro-intestinal symptoms were examined for *H. pylori* genes*, H. equorum* or both. In addition, fecal samples of 13 horses were examined for *H. equorum.* Three of the 22 samples examined with an *H. pylori*-specific assay showed the *ure* gene but not the other two genes tested. These three horses showed severe lesions in their glandular and non-glandular mucosa. However, other similarly affected horses tested negative. *H. equorum* was not found in the ten stomachs examined and only in one of the faecal samples. The outcome suggests that a urease positive microorganism may be present in the stomach of horses with severe ulceration but as no true correlation was found, the significance of this finding is doubtful.

In a study on 63 abattoir horses in Denmark, Husted et al. (2010) could not find *Helicobacter spp.* in the glandular lesions of the 36 horses with lesions. Using the

fluorescence *in situ* hybridisation (FISH) technique instead of PCR, Husted et al. made sure that they were looking for live bacteria and the technique allowed them to assess the amount and the location of the bacteria as well. One gastric erosion showed most likely *Escherichia fergusonii* both on the mucosal surface and intraepithelially. The authors could not tell whether the infection was primary or secondary. Based on their findings the authors concluded that in a normal equine stomach the glandular region harbors very little bacteria and therefore the presence of moderate or high numbers of bacteria should be considered pathological.

In addition to *Helicobacter spp.*, the bacterial community of the equine stomach has been studied in its entity. Al Jassim et al. (2008) examined at post mortem the gastric mucosa from both healthy and ulcerated non-glandular and glandular parts of 20 horses. They found that in ulcerated areas the mucosa harboured a less diverse group of bacteria than the healthy mucosa. The main bacterial groups they were able to culture from the samples were closely related to bacterial species of the genera *Lactobacillus*, *Streptococcus*, *Clostridium*, *Prevotella*, *Pseudomonas* and *Propionibacterium.* The minor groups cultured were closely related to *Escherichia, Actinobacillus, Moraxella, Rhodococcus, Veillonella, Legionella* and *Eubacterium*. The authors concluded that ulceration might lead to "good" bacteria losing their ability to adhere to the mucosa giving way to opportunistic pathogens, which could colonise the ulcers.

A more recent study on the equine gastric microbiota was conducted by Perkins et al (2012). Samples were taken from six horses during endoscopy and three horses *post mortem*. Using a combination of 16S rRNA bacterial tag-encoded pyrosequencing and FISH the team showed that, just like it has been shown for humans and other mammals, the dominant bacterial phyla of the equine gastric mucosa of these nine horses were *Firmicutes, Proteobacteria* and *Bacteroides* but there were significant variations between the horses. They also showed that certain species of *Lactobacillus* were present in the gastric mucosa of healthy horses. *Lactobacillaceae* and *Streptococcaceae* were found tightly adhered to the intact mucosal surface and to ulcerated mucosa suggesting that they were part of the gastric microbiota. No *Helicobacter spp.* were found in the mucosa of the six healthy horses.

The use of non-steroidal anti-inflammatory drugs (NSAIDs) has been linked to gastric lesions especially in the glandular part of the equine stomach (MacAllister et al. 1993, Monreal et al. 2004). However, in these studies the respective drugs (phenylbutazone, flunixin meglumine and ketoprofen in one study and phenylbutazone and suxibuzone in the other) were used at 2-3 times higher dosages than recommended by the manufacturer. In studies using recommended dosages of meloxicam, phenylbutazone and suxibuzone no statistically significant visible changes of the gastric mucosa have been shown (Andrews et al. 2009, Noble et al. 2012).

Several studies have proven that both the quality of feed and feeding practices have an influence on the formation of gastric ulcers. A study conducted on 201 horses in Denmark analysed several risk factors associated with EGUS. Unfortunately, although the authors examined both the nonglandular and the glandular part of the stomachs, the results are not presented separately for the glandular stomach. The study showed that if only straw and no or very little hay or haylage was fed, the likelyhood of gastric ulcers grade ≥ 2 in any part of the stomach increased by 4.4 times or 5.7 times depending on the way starch intake was measured. If starch intake per day was more than 2g/kg bwt the likelihood of gastric ulcers ≥ 2, glandular or nonglandular, was two times higher. And on a meal basis if it was 1-2g/kg bwt per meal the likelihood increased 2.6 times. If forage was not fed *ad libitum* the risk of nonglandular ulcers increased by 3.9 times (Luthersson et al. 2009b). Previous studies have shown that feed deprivation does not seem to induce glandular ulcers (Murray & Grady 2002).

As the significance of EGUS has been recognised, the number of both curative and preventive medical treatments has increased. The most widely used treatments are histamine type 2 receptor antagonists ranitidine and cimetidine and proton pump inhibitor omeprazole (Lester et al. 2005). Omeprazole is widely used to prevent the reoccurrence of gastric ulcers. However, no studies on the long-term effects especially on the glandular mucosa have been made. In human studies it has been shown that long-term usage of proton pump inhibitors increases the risk of gastric fundic gland polyposis up to four times after at least 12 months of PPI therapy (Jalving et al. 2006). Long-term omeprazole treatment has previously been shown to produce parietal cell hypertrophy and hyperplasia in humans (Driman et al. 1996).

2.4.2 Pathophysiology

The possible pathophysiological mechanisms discussed in connection with lesions in the equine gastric glandular mucosa have been derived from research in humans. The research on *Helicobacer pylori* –related species has been vivid but inconclusive. Therefore models of stress ulceration such as inadequate mucosal blood flow and an impaired mucus/bicarbonate barrier have been suggested as a possible facilitator of glandular lesions in the horse (Murray 1999, Andrews & Nadeau 1999). If mucosal blood flow is impaired, removal of residual HCl from the mucosal surface is inadequate. In addition, there is a possibility of reperfusion injury (Murray 1999). If mucus and bicarbonate barrier secretion is decreased and the barrier does not function properly, hydrogen ions may diffuse back and damage the submucosa (Andrews & Nadeau 1999).

One reason for impaired mucus and bicarbonate secretion and mucosal blood flow is prostaglandin inhibition (Murray 1999). The most thoroughly researched mechanism is inhibition due to the administration of non-steroidal anti-inflammatory drugs. Prostaglandins play an important part in the protection of the gastric mucosa. They are synthesised from arachidonic acid in a series of steps. The first step involves the enzyme cyclooxygenase (COX) which has two major isoforms. COX-1 is constitutive and plays a major role in gastromucosal protection, whereas the synthesis of COX-2 is both constitutive and induced by certain inflammatory mediators. The analgesic effect of NSAIDs is based on the inhibition of COX-1 and COX-2. The selectivity of COX inhibition of NSAIDs varies from nonspecific COX inhibition to highly selective COX-2 inhibition (Lees 2009).

As stated before, studies using recommended dosages of commonly used NSAIDs have not been able to produce any lesions in the equine gastric mucosa. However, it has been shown that even at recommended dosages the integrity of the gastric mucosa may be compromised. One recent study proved that administration of the nonselective NSAID phenylbutazone at the recommended dose rate for 14 days increased gastric permeability to sucrose, even though no ulceration of the squamous or glandular mucosa could be detected endoscopically. The relatively selective COX-2 inhibitor

meloxicam did not have such an effect even at 5 times the recommended dose (D'Arcy-Moskwa et al. 2012).

2.4.3 Diagnosis

To diagnose EGGES properly the examiner must have an endoscope of at least 2.5 m but preferably 3 m length (Murray et al. 2001b). Prior to endoscopy the horse needs to be fasted to have an unobstructed view of the glandular mucosa. It seems that even 12 hours of food deprivation is too short to view the entire glandular mucosa (Vatistas et al. 1999). To view the antrum and the pylorus, the endoscope must pass the most dependent portion of the stomach, which in some horses may prove extremely difficult. To facilitate the advancement of the endoscope, the stomach may be insufflated with air, fluid can be aspirated and biopsy forceps used to pull the antrum toward the endoscope (Murray et al. 2001b).

However, a study performed by Andrews et al. (2002) showed that even with feed deprivation of 18-24 hours, using a 2,75 m endoscope, insufflation of the stomach and flushing the mucosa with water, small glandular ulcers were missed by the endoscopist and only discovered at necropsy.

2.4.4 Treatment

To date there is only one article on the treatment of glandular lesions. Sykes et al. (2012) compared the efficacy of two different doses of omeprazole on gastric ulceration and compared the healing response of the squamous and glandular mucosa. They found that the treatment had a better effect on squamous ulcers than glandular ulcers and that glandular ulcers healed more slowly than squamous ulcers. In addition, glandular ulcers were more likely to heal or improve if squamous ulcers responded to treatment. The authors speculate that glandular ulcers may require more than 28 days of omeprazole treatment or possibly antimicrobial treatment or gastroprotectants.

2.4.5 Prevention

There is no research on the prevention of EGGES. Habershon-Butcher et al. (2012) identified several risk factors for glandular ulceration. The likelihood for glandular ulcers was higher for mares and geldings than colts, for horses with no grass turnout, horses which were in direct contact with other horses, horses that were not fed haylage,

horses that were fed unprocessed grain, horses that were infrequently fed a complete diet, horses that underwent fast exercise on fewer days of the week and for horses that went swimming. The trainer was also listed as a risk factor in this study. Unfortunately, these results have not been published in full and therefore a more thorough analysis of their significance is not possible. As the pathogenesis of EGGES is still is poorly understood, there is little evidence to rely on in the prevention of EGGES.

3 STUDY OBJECTIVES

The objectives of the experimental part of this study are to describe the glandular part of the stomach of horses with EGGES both macroscopically and histologically and to compare these descriptions with that of a normal equine glandular stomach. In addition, the gross lesions and the histopathological findings will be presented as photographs. The focus will be on the glandular part of the stomach only, and all types of lesions including non-erosive ones will be described.

4 MATERIALS AND METHODS

4.1 Horses

The horses for this study were selected from horses euthanased at the Equine Hospital of the Helsinki University Veterinary Teaching Hospital and sent to the Pathology and Parasitology unit of the Faculty of Veterinary Medicine, University of Helsinki for necropsy. The material was collected from August 2013 – February 2014. Only adult horses were included in this study.

After the horses arrived at the pathology unit, a necropsy was performed by either students of veterinary medicine or the regular staff of the unit. The stomachs were removed, opened along the greater curvature, rinsed gently with tap water and assessed macroscopically. Stomachs with either visible lesions in the glandular mucosa or no lesions at all were selected for sampling and photographed. Altogether five stomachs were sampled. Two stomachs showed gross lesions and three appeared normal. However, only one normal-looking and one with lesions were suitable to be analysed for study purposes. The other stomachs showed severe signs of autolysis due to delays between euthanasia and necropsy.

The two horses selected for this study were both Finnhorses. The control horse (referred to as horse A in the remainder of the study) was a 16-year-old gelding that used to be used for trotting, but was no longer in active training. It was euthanased because of acute colic symptoms which did not respond to analgesic medication. It had been treated with flunixin meglumine (13 ml Flunixin 50 mg/ml i.v.) prior to euthanasia.

The horse with lesions in the glandular stomach (horse B) was a 2-year-old stallion trotter that was in training, which was euthanased due to severe neurological symptoms. Necropsy revealed signs of possible chronic meningitis and bilateral neutrophilic vasculitis of the femoral arteries and veins. Vascular damage was also present in the lungs and brain. The aetiology of the vascular lesions could not be identified but may have been bacterial. During the acute phase of illness, which lasted for two days, the horse was treated repeatedly with flunixin meglumine with a dose rate exceeding the recommended dosage of 1,1 mg/kg BWT once a day (Table 1).

Tab. 1 Flunixin meglumine dosages of horse B before euthanasia

	Interval to previous (h)	Dose mg/kg BWT
Dose 1 (i.v.)	-	1.1
Dose 2 (p.o.)	6	0.9
Dose 3 (p.o.)	16	0.9
Dose 4 (p.o.)	10	0.9
Dose 5 (i.v.)	11.5	1.1

4.2 Histopathology

Samples were taken from the cardia, the margo plicatus, the glandular fundus, and the pylorus (one 1cm^2 sample from each area). In the case of glandular lesions, samples were also taken from visible lesions. The samples (including all layers of the stomach) were obtained using forceps and a scalpel blade. The tissues were fixed in 10% neutral buffered formalin for 96-120 hours, cut, placed in plastic cassettes, dehydrated and cleared by a tissue processor overnight. The next morning they were embedded in paraffin. The cold paraffin block was cut with a microtome into 4 µm sections. After processing in warm water the sections were picked up on glass microscopic slides, dried and fixed. They then underwent routine staining with Hematoxylin and Eosin. The histopathologic evaluation was done by an experienced pathologist.

5 RESULTS

The stomach of horse A (Fig.3) was macroscopically normal with the exception of some nodules in the squamous mucosa at the *margo plicatus* interpreted as hyperplastic nodules, a common change in the *margo plicatus* and squamous part of the stomach seen in older horses, as well as a few reddish areas in the fundic and pyloric regions, interpreted as artifacts caused by improper handling at necropsy. The stomach had been removed and opened by third-year students participating in their necropsy exam.

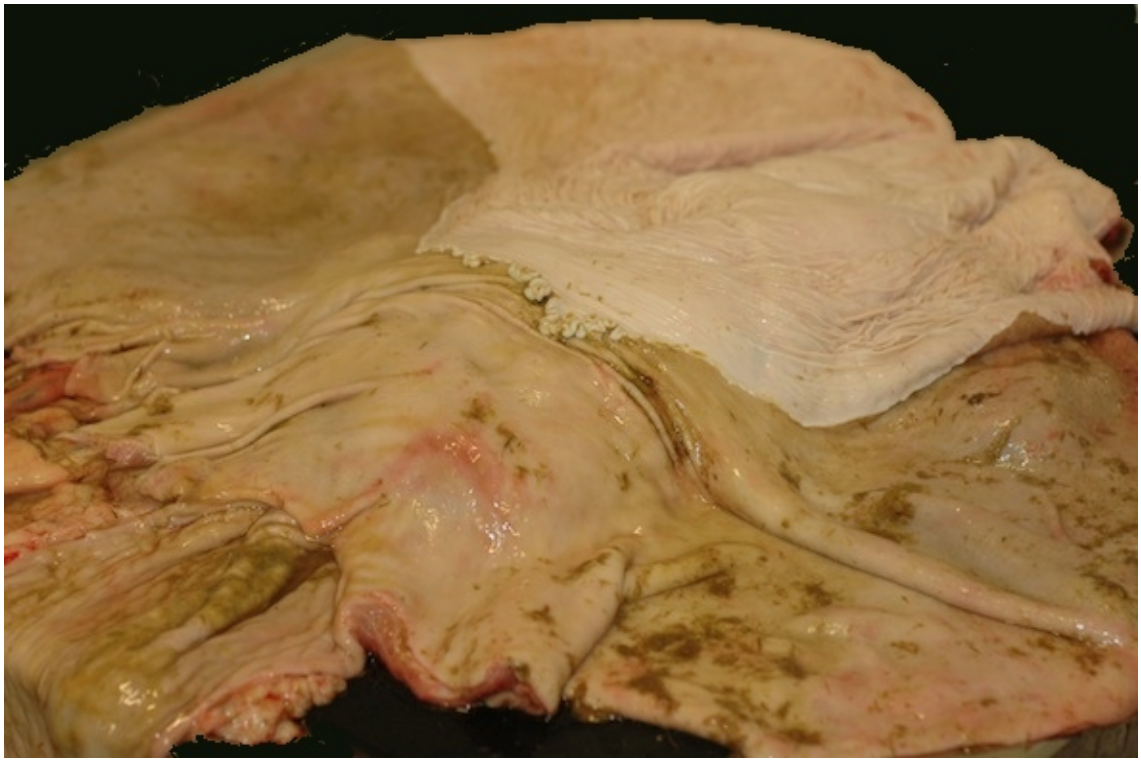

Fig. 3 Normal equine stomach, horse A

Microscopically, the nodules proved to be mild, focal nodular squamous proliferations. However, there was no evidence of glandular metaplasia or dysplasia. Except some mild diffuse hyperaemia, the fundic gland region appeared normal (Fig.4). The *muscularis mucosa* had a thickness of 0.5mm. There were no changes present in the epithelium but the samples were not fresh enough to rule out mild changes such as erosions.

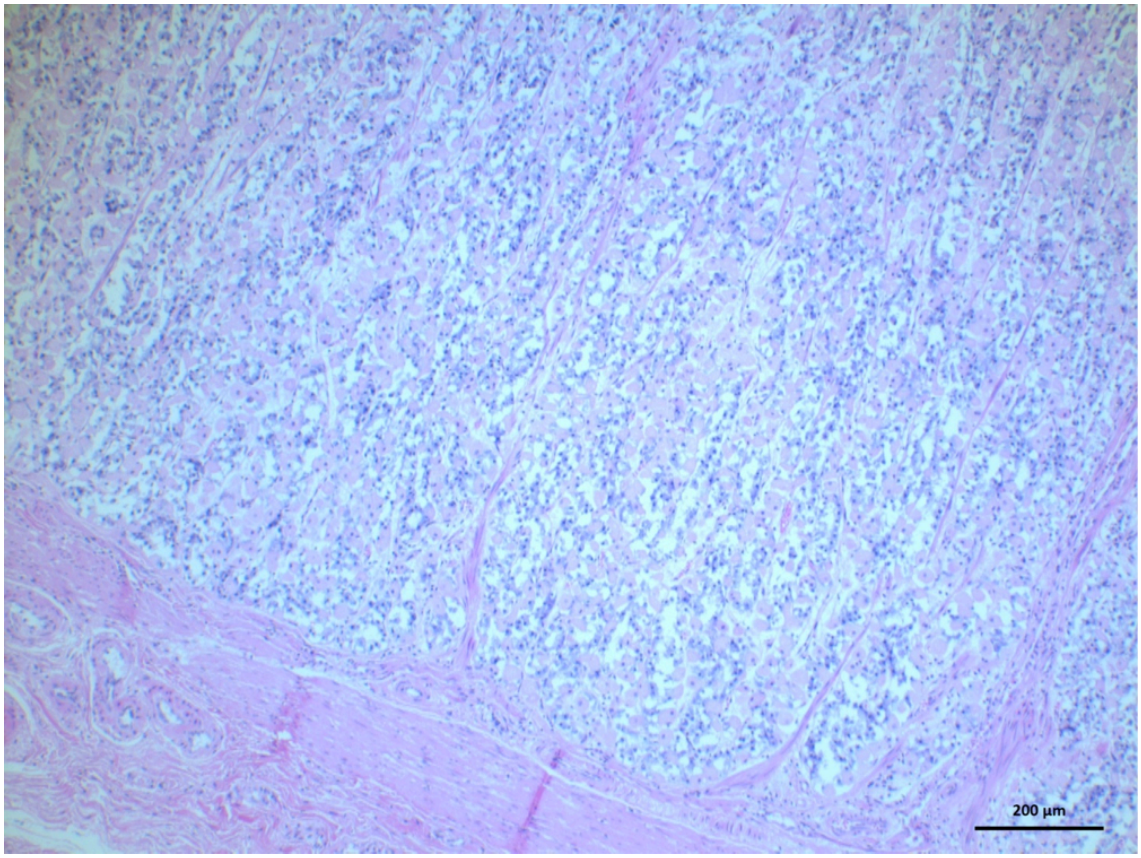

Fig. 4 Horse A. Normal fundus. Regularly distributed fundic glands and minimal amount of connective tissue. H&E.

In the pyloric region the mucosa was intact and the gastric pits were regular. Most of the gastric glands were mucus-secreting glands. The amount of intertubular connective tissue in the *lamina propria* was minimal and the glands were regularly distributed. Small numbers of lymphocytes and macrophages were scattered between the glands which is a normal finding in this area.

The gross changes in the glandular stomach of horse B (Fig.5) were marked. Along the greater curvature the glandular fundus was moderately hyperaemic (Fig.6) with multifocally distributed erosive lesions (Fig.7) and mild diffuse oedema of the gastric wall. Multifocal 0.2-0.8cm ulcerations and streaks of hyperaemia were visible in the pyloric region (Fig.8).

Fig. 5 Stomach of horse B showing lesions in fundic and pyloric regions

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Fig. 6 Marked hyperaemia along greater curvature of stomach B.

Fig. 7 Glandular fundus of horse B showing moderate hyperaemia and diffuse erosive lesions.

Fig. 8 Pyloric region of horse B with ulcerative lesions.

Histology showed mild irregularity in distribution of the glands in the fundic region resulting from expansion of the *lamina propria* by an increased amount of connective tissue, causing separation of the glands in uneven columns (Fig.9). The *muscularis*

mucosa was 1mm thick. In sections closer to the ulcer there was marked congestion and fibrosis in the *lamina propria* (Fig.13).

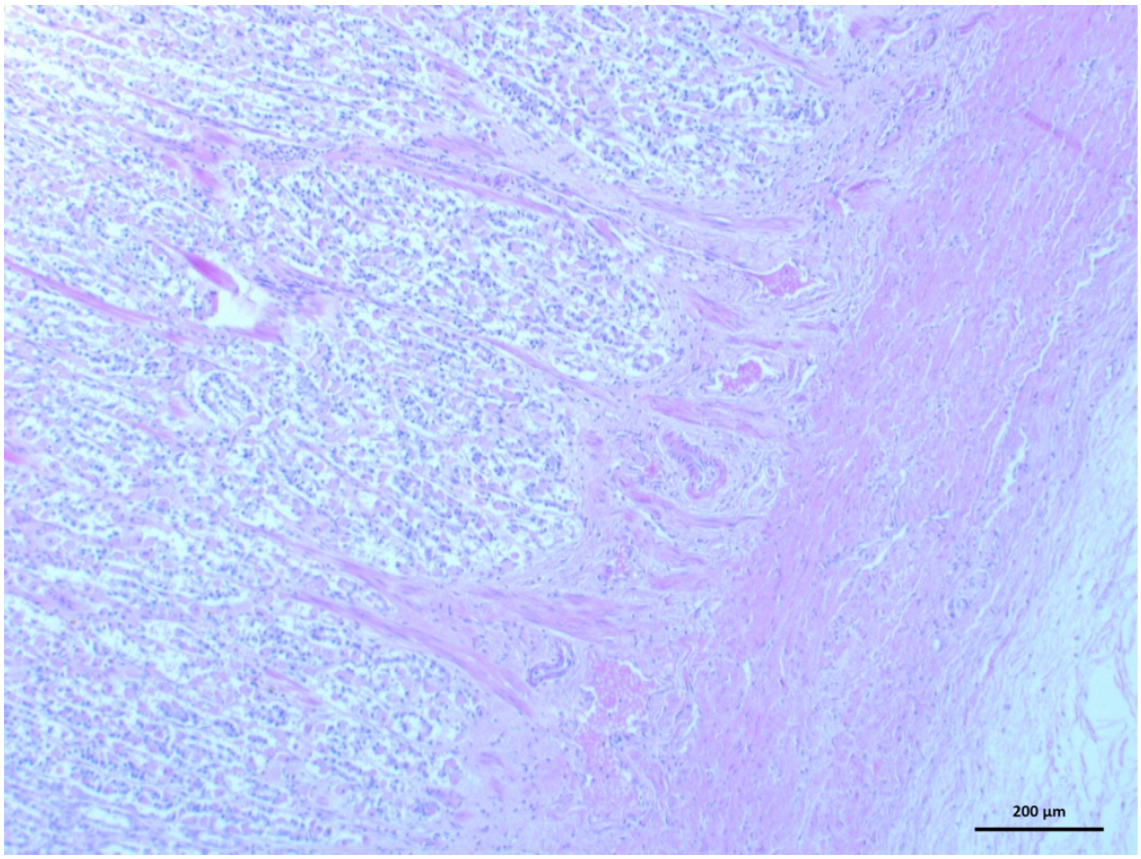

Fig. 9 Horse B: Fundus. Fibrosis in lamina propria and mild congestion. H&E.

In the pyloric region, multiple 0.5-1cm ulcerations in the mucosa were characterized by a complete loss of epithelium, replaced by fibrin and bacterial colonies of Gram-positive cocci (Fig.10). The bacterial colonies were restricted to the surface of the mucosa. In areas adjacent to the ulcers, the *lamina propria* and submucosa were multifocally expanded by an increased amount of fibrous tissue (Fig.12). In the underlying submucosa and *muscularis externa* there were vast areas of necrosis resulting in marked expansion by fibrin, moderate numbers of neutrophils, macrophages and fewer eosinophils. Fibrin thrombi filled the submucosal vessels, and fibrinoid necrosis was present within some vascular walls. (Fig.11) This was interpreted as an extension of the necrosis of the submucosa, and not a primary vascular-targeted inflammation. Other sections of the pyloric region distant from the lesions exhibited mild to moderate hyperaemia, submucosal oedema and mild fibrosis of the *lamina propria*.

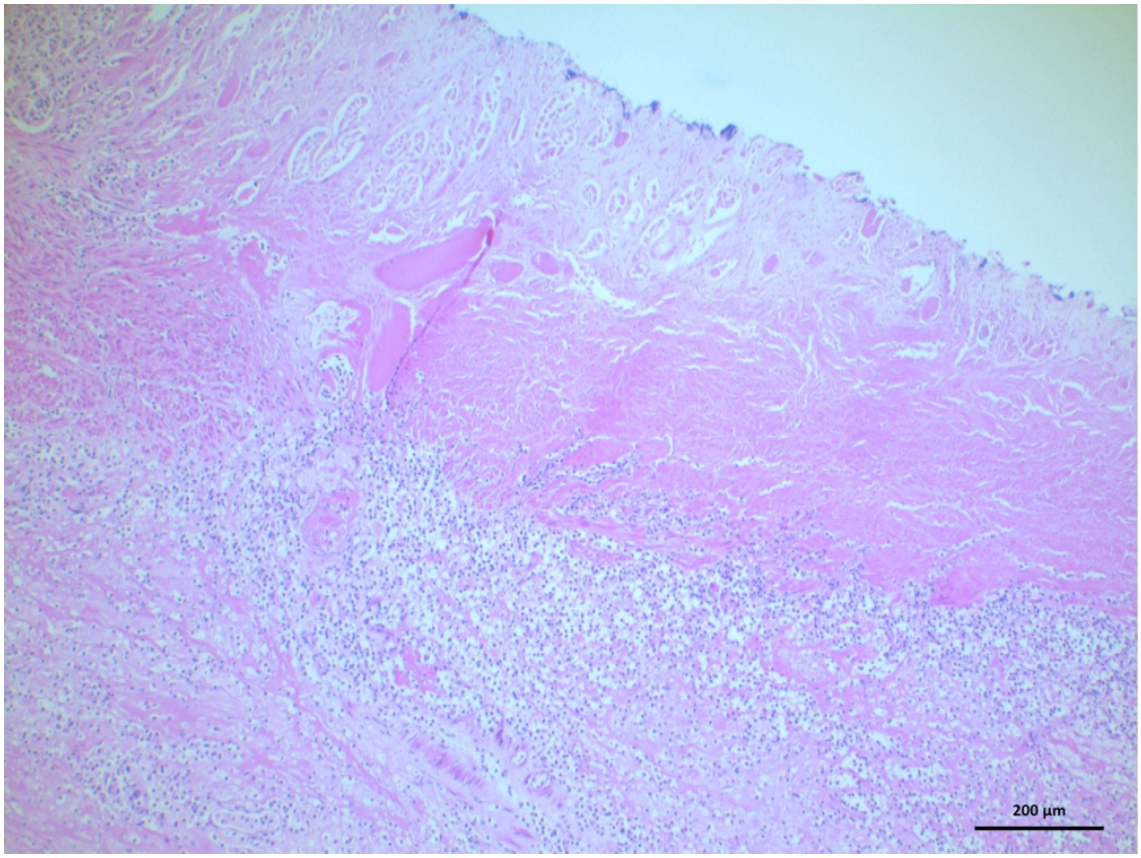

Fig. 10 Horse B: Area of extensive ulceration. Complete loss of epithelium. Mucosa replaced by fibrin and inflammatory cells. Bacterial colonies on mucosal surface. H&E.

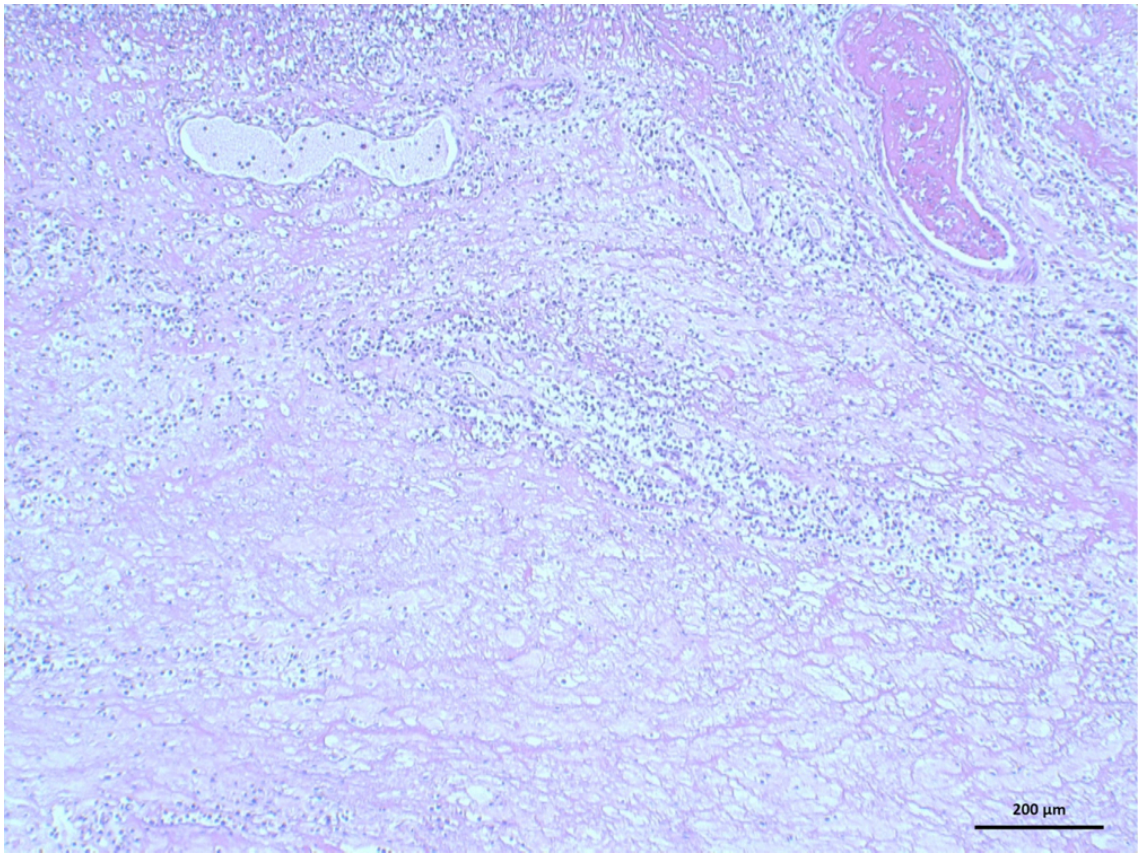

Fig. 11 Horse B: Complete necrosis of submucosa underlying ulcer. Vasculitis and fibrinoid necrosis of the vascular walls. Note fibrin thrombus in arteriole on the right. H&E.

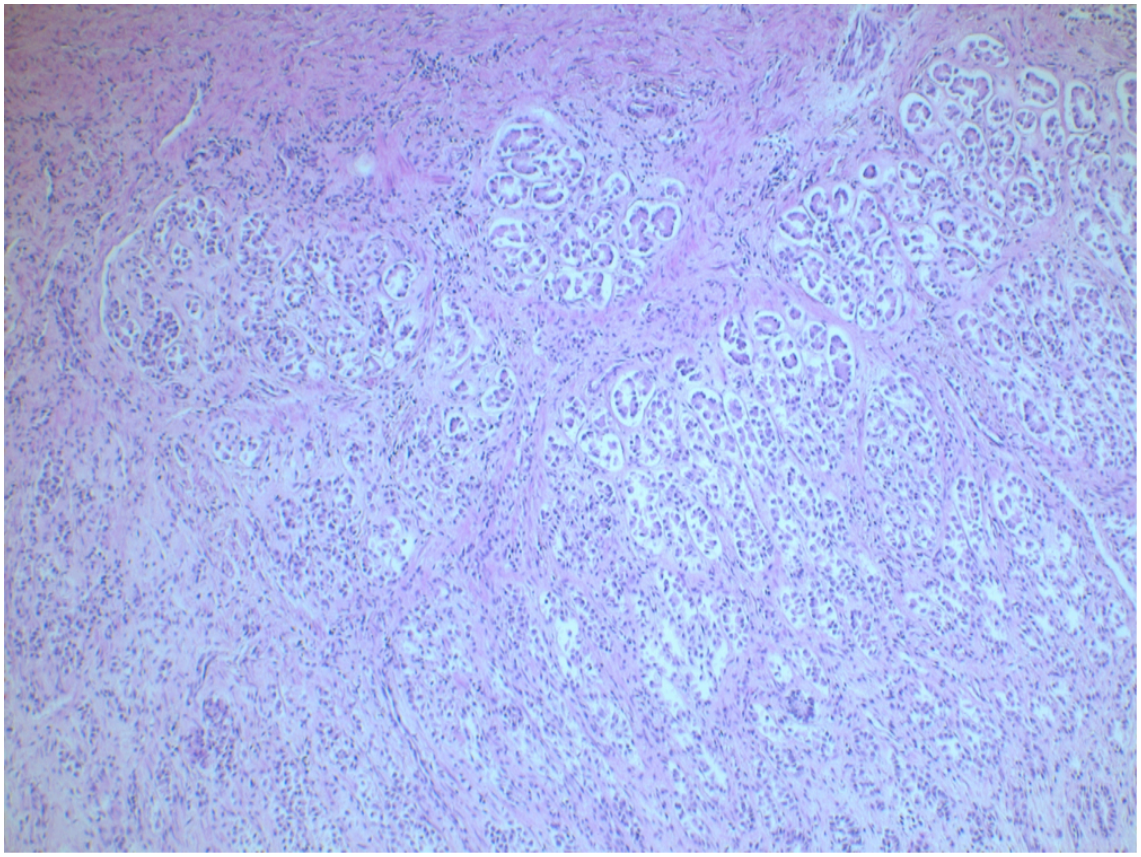

Fig. 12 Pyloric region adjacent to ulcerated area. Moderate to marked fibrosis in lamina propria separating glands. H&E X50.

When compared to the pyloric area of the control case the differences are clear. The colonies of Gram-positive cocci, the necrosis of the submucosa and muscularis externa as well as the moderate numbers of inflammatory cells clearly suggest a multifocal moderate chronic active ulcerative gastritis in the pyloric area of horse B. In contrast the gastric glands in the normal pyloric region were regular and no excessive fibrous tissue was present between the glands. The normal *lamina propria* was infiltrated by small numbers of lymphocytes and macrophages, representing normal cell population of the gastric mucosa.

The fundic gland region was mildly hyperaemic in both horses but only in horse B were the fundic glands mildly irregular as the *lamina propria* had expanded due to an increased amount of connective tissue. The *muscularis mucosa* was twice as thick in horse B as in horse A.

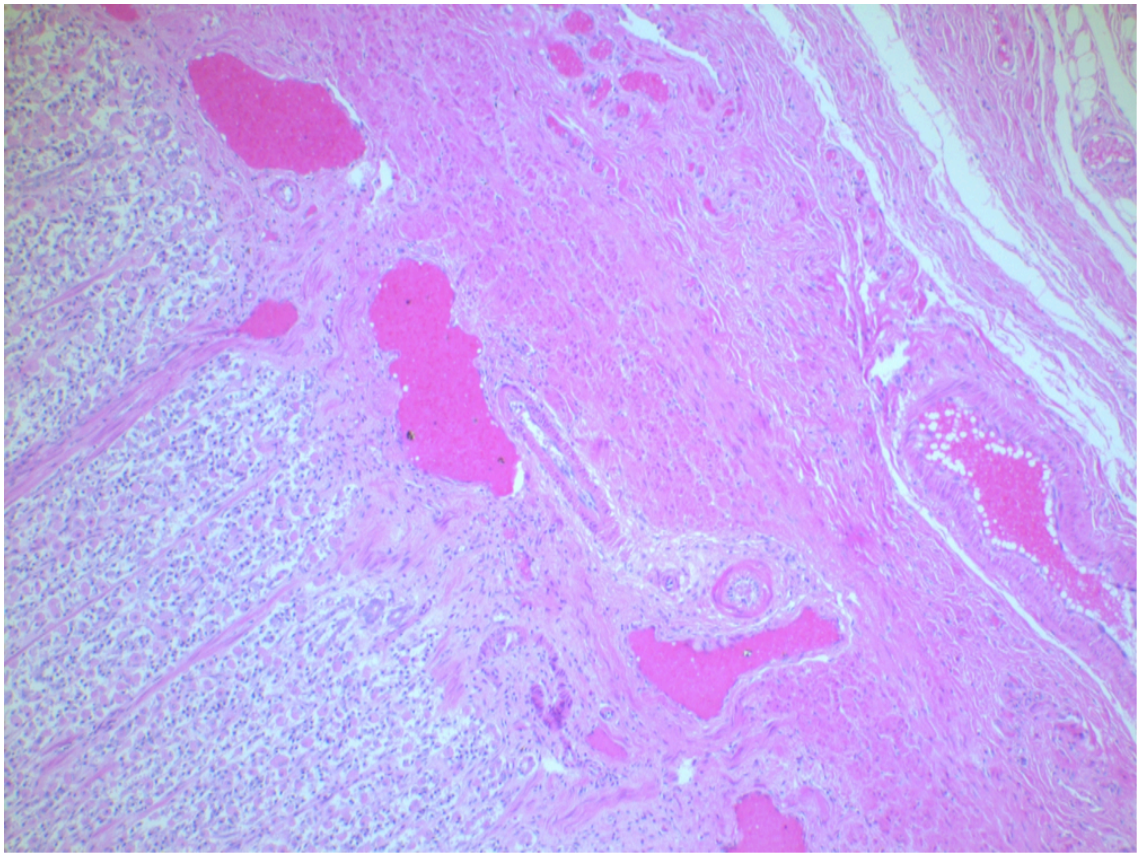

Fig. 10 Horse B: Fundus. Marked congestion and fibrosis in lamina propria. Area closer to ulcer. H&E X50.

6 DISCUSSION

6.1 Histopathology

To date only one study on the histopathology of glandular lesions in the equine stomach exists. Martineau et al. (2009a) examined samples from 21 stomachs. In gross examination, they found hyperaemia in 10/21 stomachs which histologically appeared either normal or as congestion of superficial arteries. No vasculitis or necrosis of the vascular wall was reported in the glandular part of any of the stomachs. Focal erosions were found in twelve stomachs in all regions of the glandular stomach but most frequently in the pyloric area. Histologically, these erosions comprised maximally half the depth of the glandular tissue. In one case gross examination indicated ulcers in all glandular regions and in three cases in the pyloric region. However, none of these lesions were histologically ulcers as the mucosa was not lost entirely and there was no penetration through the *muscularis mucosa*. All samples were stained with Warthin Starry silver stain but no *Helicobacter*-like organisms were detected (Martineau 2009a).

In connection with their study, Martineau et al. (2009b) made an attempt at developing a scoring system for histological descriptions. Using this scoring system for their material, they diagnosed 21/22 horses with chronic gastritis. Eleven of these had chronic active gastritis. In 14 cases the gastritis was classified as mild, in 5 cases as moderate and in one case as marked depending on the degree of cellular infiltrate.

In contrast to the stomachs examined by Martineau et al. (2009b), the stomach of horse B showed multifocal deep ulcerations which extended into the superficial part of the *tunica muscularis*. The most severe lesions were located in the pyloric region. Multiple colonies of Gram-positive coccoid bacteria were found on the surface of these lesions. It would have been beyond the scope of this study to try and identify these bacteria. Thus it is impossible to say whether these bacteria were part of the normal microbiota found in the equine stomach and identified in previous studies.

There are several factors in the background of horse B that make the assessment of the significance of these lesions in regard to EGGES extremely challenging. First of all, the horse suffered from meningitis and vasculitis with possible bacterial origin. Vascular

damage was not only seen in the brain but in the femoral arteries and veins and in the lungs. Infectious meningitis is very rare in adult horses. It is known to spread hematogenously (Pellegrini-Masini & Livesey 2006). However, according to the analysis of the histopathological samples from the glandular stomach of horse B, the necrosis of the vascular walls in the submucosa was the result of an expanding inflammation in the submucosa and not a primary vasculitis spreading into adjacent tissue.

Secondly, the horse had received flunixin meglumine at the recommended dose but twice daily as opposed to the recommendation of once a day. It has been shown that if flunixin meglumine is administered at the recommended dose every eight hours, it may cause lesions in the glandular stomach within 7 days (MacAllister & Sangiah 1993). Whether administration every 6-16 hours for two days is enough to cause lesions in the glandular stomach can only be speculated on.

Judging from the chronic nature of the gastric lesions in horse B they may have started to form before the onset of acute illness. However, there is no gastroscopy result to prove this. The horse had been in pasture with other stallions and not in active training for three months before it became ill. Even if the gastritis already existed when the horse was hospitalised, it is possible that the stress of acute illness and the NSAID administration aggravated the lesions.

6.2 Limitations of the study

The original plan was to collect samples from as many horses with EGGES as possible to cover all possible range of lesions. Unfortunately, scheduling problems, improper timing and delays in transport of euthanased horses led to a situation were horses were missed, the number of horses coming to pathology was at times very low or the stomachs suffered from autolysis too severe to be used for study purposes. In the end, the only suitable stomach for control purposes was from a horse that had been euthanased due to gastrointestinal symptoms, which may well have been the reason, why the stomach was not entirely normal histologically. In addition, only one stomach with ulcerative lesions could be analysed. Therefore the results of this study cannot be generalised in any way. It does not give a full description of the various lesions seen in EGGES. It only scratches the surface.

For this study stomachs had to be used that were handled by students unaware of the ongoing study. In an ideal case, the stomachs would have been removed from the horses right after euthanasia by trained pathologists aware of the study protocol. There were also other flaws in the study protocol. For future studies it would be advisable to make a gross description of the stomach in addition to photographing it. A map of the location of the lesions sampled for histology would have been helpful as well. The treatment of the horses before euthanasia should have been checked and horses with a possible systemic infection excluded from the study.

6.3 Future studies

As this study has shown, much remains to be discovered about the aetiology, pathogenesis, risk factors, treatment and prevention of EGGES. As there still is very little material on the histopathology of EGGES, the present study should be repeated with a broader spectrum of horses paying attention to the validity of the collected data. As the prevalence of EGGES in Finland has not been studied yet, and there seem to be regional differences in prevalence (Habershon-Butcher et al. 2012), this would be an interesting topic to explore in connection with the histopathology study.

Apart from that, it has yet to be established whether glandular lesions are connected with similar symptoms as those that have been reported in connection with EGUS and whether there is variation in symptoms correlating to the variation in lesion type. For proliferative lesions in the glandular part it would be interesting to find out, if long-term treatment with omeprazole has similar consequences in horses, as it seems to have in humans (Driman et al. 1996, Jalving et al. 2006). The last word has not been spoken on possible bacterial involvement either. More research in this field is needed, before the usefulness of antimicrobial treatment (Sykes et al. 2012) can be evaluated.

7 CONCLUSION

The aim of this study was to contribute to the understanding on EGGES. The review of the existing literature showed that even though some points have already been covered, plenty still remains to be explored. Even the basic physiology of the equine stomach has not been thoroughly investigated.

Regarding the aetiology of EGGES, a bacterial aetiology, whether *Helicobacter spp.* or other, has been the focus of most vivid research but the results are still inconclusive; also, there is no evidence in the literature that proper NSAID use on its own would be the determining factor for EGGES but it could well be one factor disrupting the function of the protective barrier of the glandular mucosa. It is quite clear that acid exposure only plays a role when the protective barrier no longer functions properly. The role of duodenal reflux of bile acids as a compromising factor should be explored more thoroughly. There seems to be a connection between feeding regimes and EGGES even though feed deprivation has not been identified as a risk factor. The influence of stress and training should also be examined. All in all, summarizing all the aspects covered in the literature, a multifactorial aetiology of glandular erosions and ulcers seems very likely. Factors disrupting the function of the mucus-bicarbonate barrier expose the underlying cells to the acidic contents of the stomach which leads to the formation of erosions and ulcers as the mucosa is no longer protected. When it comes to proliferative lesions, the literature is even scarcer. It is possible that the aetiology of these lesions differs from that of ulcerative lesions at least in some aspects. The role of long-term omeprazole treatment should be considered.

The empirical part of this study set out to describe the histopathology of EGGES lesions and to compare them to the histology of a normal equine stomach. Unfortunately, only one case was suitable for the study. The glandular lesions in that horse were shown to be more severe than any described in the literature before. However, the influence of the original illness and treatment of the horse could not be assessed. The study showed that in addition to erosions, hyperaemia, glandular atrophy, dysplasia and hyperplasia described by Martineau, severe ulcers and vasculitis can also be seen in the glandular stomach of horses.

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